Colloquium

Therapeutic vaccines in autoimmunity

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Similarly to prophylactic vaccines whose purpose is to prevent infectious diseases, therapeutic vaccines against autoimmune diseases are based on their similarity to the putative causes of the disease. We shall describe here two such examples: a copolymer of amino acids related to myelin basic protein, in the case of multiple sclerosis, and a peptide derived from the nicotinic acetylcholine receptor (AChR), in the case of myasthenia gravis (MG). Copolymer 1 (Cop 1, glatiramer acetate, Copaxone) is a synthetic amino acid random copolymer, immunologically cross-reactive with myelin basic protein and suppresses experimental allergic encephalomyelitis in several animal species. Cop 1 slows the progression of disability and reduces relapse rate in exacerbating-remitting multiple sclerosis patients. It was approved by the Food and Drug Administration in 1996, and today is used by tens of thousands of patients. Cop 1 is a potent inducer of T helper 2 (Th2) regulatory cells in mice and humans, and Th2 cells are found both in the brains and spinal cords of Cop 1-treated mice. MG and experimental autoimmune MG are T cell-regulated, antibody-mediated autoimmune diseases. Two peptides, representing sequences of the human AChR α-subunit, p195-212 and p259-271, are immunodominant T cell epitopes in MG patients and in two strains of mice. Altered peptide ligand, composed of the tandemly arranged two single amino acid analogs, inhibits *in vitro* and *in vivo* MG-associated autoimmune responses. The active suppression is mediated by the CD4$^+$CD25$^+$ immunoregulatory cells and is associated with the down-regulation of Th1-type cytokines and the up-regulation of the secretion of IL-10 and the immunosuppressive cytokine, transforming growth factor β.

Vaccines are prophylactic in the sense that they are administered to healthy individuals to prevent a disease. Nevertheless, there is a growing trend to use vaccines to alleviate the suffering of those already having a disease. Great effort is being devoted to develop vaccines against tumors, AIDS, hepatitis, tuberculosis, Alzheimer's disease, Huntington disease, etc. What is characteristic for a vaccine is its specificity. You do not have one vaccine against all kinds of different viruses or bacteria. For every troublemaker, there is a "molecular cousin," close enough in its chemical composition to lead to an immune response cross-reactive with the troublemaker but harmless biologically because the danger of the original virus or bacterial toxin has been knocked out. This situation is analogous in the case of therapeutic vaccines. At least one therapeutic vaccine, copolymer 1 [(Cop 1), or glatiramer acetate (GA)] for the relapsing-remitting form of multiple sclerosis (MS), is being used by many tens of thousands of patients.

We shall describe here a therapeutic vaccine against the relapsing-remitting form of MS, as well as a candidate vaccine for myasthenia gravis (MG). In both cases, bystander suppression plays an important role.

MS

Preclinical Studies. In our early studies of synthetic polypeptide antigens of special interest was the immune response to lipid components, which was not easy to either elicit or investigate because of solubility problems. However, conjugates in which synthetic lipid compounds were attached onto synthetic copolymers of amino acids elicited a specific response to lipids such as cytolipin H, which is a tumor-associated glycolipid (1), or sphingomyelin (2). Furthermore, we demonstrated that both the sugar and lipid components of such molecules contributed to their immunological specificity. The resultant anti-lipid antibodies were capable of detecting the corresponding lipids both in water-soluble systems and their physiological milieu. This observation was fascinating because it gave us a glimpse into some disorders involving lipid-containing tissue and consequently led to our interest in demyelinating diseases, namely, disorders in which the myelin sheath, which constitutes the lipid-rich coating of all axons, is damaged, resulting in various neurological dysfunctions. We, thus, thought that experimental allergic encephalomyelitis (EAE) caused by myelin basic protein (MBP) might actually be induced by a demyelinating lipid and that the positively charged MBP might serve only as a carrier for an acidic lipid (e.g., phospholipid). We prepared several positively charged copolymers of amino acids and tested whether we could induce EAE when the copolymers were administered into experimental animals (guinea pigs and rabbits) in complete Freund's adjuvant, similarly to the successful administration of MBP, but we failed. On the other hand, the injection of several positively charged amino acid copolymers in aqueous solution into mice, rabbits, and guinea pigs resulted in efficient suppression of the onset of the disease EAE (3–5). Later on, we could suppress the actual disease in rhesus monkeys and baboons (5, 6). The Cop 1 we primarily used, now called GA or Copaxone, is composed of a small amount of glutamic acid, a much larger amount of lysine, some tyrosine, and a major share of alanine. Thus, its overall charge is positive. To our pleasant surprise, there is a significant immunological cross-reaction [both at the antibody level (7, 8) and T cell level (9, 10)] between Cop 1 and the MBP. Interestingly, when an analog of Cop 1 made from D-amino acids was tested, it had no suppressing capacity nor did it cross-react immunologically with the basic protein (11). Cop 1 is not generally immunosuppressive; and it is not toxic. Actually, it is not helpful in any other autoimmune disease except MS and its animal model EAE.

GA was demonstrated to suppress EAE induced by MBP in a variety of species: guinea pigs, rabbits, mice, and two species of monkeys (rhesus monkeys and baboons) (Table 1). In contrast to rodents where GA inhibits the onset of the disease, in primates it was used as treatment of the ongoing disease. A remarkable degree of suppression of EAE by GA was demonstrated in all

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Abbreviations: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; MG, myasthenia gravis; EAMG, experimental autoimmune MG; APL, altered peptide ligand; AChR, acetylcholine receptor; GA, glatiramer acetate; Cop 1, copolymer 1; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein; PBL, peripheral blood lymphocytes; MMP, matrix metalloproteinase; PLC, phospholipase C; Th, T helper; TGF, transforming growth factor.

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Table 1. Suppression of EAE induced by different encephalitogens and in various species by GA

Encephalitogen	Disease type	Species	Inhibition by GA, %
MBP	Acute EAE	Guinea pigs	70
		Rabbits	73
		Mice (SJL/JXBalb/C)	67
		Rhesus monkeys	80
		Baboons	78
Spinal cord homogenate	Chronic-relapsing EAE	Guinea pigs	58
		Mice	55
PLP 139-151	Chronic-relapsing EAE	Mice (SJL/JXBalb/C)	100
PLP 178-191	Chronic EAE		86
MOG 35-55	Chronic EAE	Mice (C3H.SW)	50

species studied, although different encephalitogenic determinants of MBP are involved in disease induction in the different species. Furthermore, GA was effective in suppressing the chronic relapsing EAE, a disease that shows a closer resemblance to MS, that can be induced by either spinal cord homogenate or encephalitogenic peptides derived from proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) (Table 1). Thus, the suppressive effect of GA in EAE is a general phenomenon and is not restricted to a particular species, disease type, or the encephalitogen used for EAE induction.

GA exhibits a very rapid, high, and efficient binding to different MHC class II haplotypes on living murine and human antigen-presenting cells (12). Processing of GA is not required before its binding to MHC molecules (13). GA was also shown to interact with purified HLA-DR molecules (DR1, DR2, and DR4) with high affinity (14, 15). Furthermore, the fraction of GA that was eluted from the different DR molecules had a similar amino acid composition to that of intact GA, indicating that the same types of determinants are involved in the binding. As a result of its high and efficient binding to MHC class II molecules, GA is capable of competing for binding with MBP and other myelin-associated proteins, such as PLP and MOG. Moreover, GA can efficiently displace MBP-, PLP-, and MOG-derived peptides from the MHC binding site, whereas it could not be displaced once bound to the MHC by these antigens (16).

In vivo studies have demonstrated that GA-treated animals develop GA-specific T suppressor cells in the peripheral immune system (also called T regulatory cells, or Tregs). These cells can adoptively transfer protection to EAE (17). Furthermore, T suppressor cell hybridomas and lines could be isolated from spleen cells of mice and rats rendered unresponsive to EAE by GA. These T suppressor cells were characterized as T helper (Th)2/3 type cells secreting antiinflammatory cytokines such as IL-4, IL-10, and transforming growth factor (TGF) β but not Th1 cytokines (18).

Clinical Studies. Two recent comprehensive review articles, dedicated almost exclusively to this subject, described in detail the various clinical trials that led to the approval of GA as a drug for the treatment of MS and its evaluation (19, 20). In the following we will relate to these clinical studies briefly and focus on additional findings that were reported more recently. After a couple of early clinical trials, it was clear that GA showed efficacy in treating patients with the relapsing-remitting disease. In three randomized double-blind trials [including a 2-year pilot trial (21), a larger American 2-year pivotal trial (22), and a 9-month European/Canadian (23)], GA, at a dose of 20 mg once daily, administered s.c. in patients, was significantly more effective than placebo for the respective primary endpoint of each trial (proportion of relapse-free patients, relapse rate, and number of enhancing lesions on MRI scans).

For patients receiving GA compared with those receiving placebo in the two larger comparative studies, the mean relapse rate (covariate adjusted) as study endpoint was 29% lower in the large American trial (where relapse rate was the primary endpoint) and 33% lower in the European/Canadian study (where relapse rate was the tertiary endpoint). In the pilot trial, GA recipients had a mean relapse rate 78% lower, and they were more than twice as likely to be relapse free than placebo recipients.

GA decreased activity and burden of disease, as assessed by analysis of MRI scans, in patients enrolled in the European/Canadian study (23) where certain MRI measures were the primary and secondary endpoints. For the primary outcome measure, patients in the GA-treated group demonstrated 29% fewer gadolinium-enhancing CNS lesions (areas of acute inflammation representing disruption of the blood–brain barrier) than patients in the placebo group. For secondary MRI outcomes, GA showed significantly greater lesion reductions (ranging from 30% to 82.6%) than placebo.

Progression to sustained disability, as measured by the Kurtzke expanded disability status scale, was secondary endpoint in the two long-term trials. Patients with relapsing-remitting MS treated with GA in the pivotal American trial were significantly more likely to experience reduced disability, and placebo recipients were more likely to experience increased disability. The overall disability status was also significantly improved in this trial, although the change was modest. The pilot trial showed positive trends in delaying the onset of worsening of disability, although it did not have adequate statistical power to evaluate this outcome.

The beneficial effect of GA persisted far beyond the duration of the trials. Thus the relapse rate for an extension period (up to 35 months) of the American trial suggested a sustained benefit for patients receiving GA vs. those receiving placebo (24). Furthermore, the annualized relapse rate for patients who had received GA throughout the 6-year active-treatment extension phase was 72% less than the annualized relapse rate at study entry ($\rho = 0.0001$) (25). Patients receiving GA for 8 years (26) had an annualized relapse rate for the eighth year of 0.16 (equivalent to one relapse in 6 years) compared with a baseline annualized rate of 1.49 (based on the rate for the 2-year pretreatment period; Fig. 1).

As for its safety profile, from all of these clinical trials it emerges that GA is well tolerated. The most commonly reported treatment-related adverse events are localized injection-site reactions and transient postinjection systemic reactions. GA is not associated with the influenza-like syndrome or the formation of neutralizing antibodies that are reported in patients treated with IFN-β. Antibodies to GA do not interfere with its biological functions and therapeutic efficacy (27). In a recent review, it was concluded that, as the most

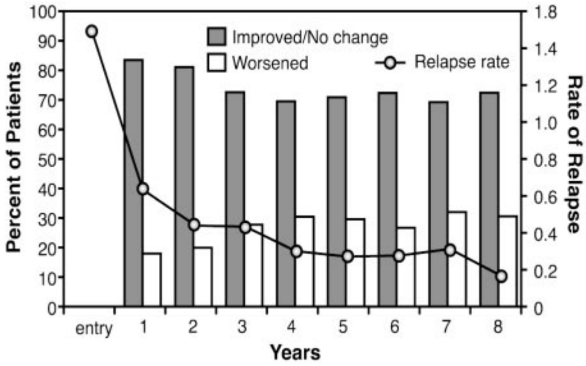

Fig. 1. Results of long-term (8 years) prospective open trial of GA for relapsing-remitting MS. Yearly expanded disability status scale change by year of study. All patients received GA (26).

common adverse effects were mild and consisted mainly of injection-site reactions, and as antibodies to GA do not interfere with clinical effects, GA has an excellent risk-benefit profile in relapsing-remitting MS patients (28).

In three different clinical trials we investigated humoral and cellular immune responses in MS patients treated with GA (29). All patients (130) developed Cop 1-reactive antibodies, which peaked at 3 months after initiation of treatment, decreasing at 6 months and remaining low. IgG1 antibody levels were 2- to 3-fold higher than those of IgG2. The proliferative response of peripheral blood mononuclear cells to Cop 1 was initially high and gradually decreased during treatment. Antibodies and T cell responses to MBP were low and did not change significantly during the treatment. The preferential production of IgG1 over IgG2 antibodies may indicate that Th2 responses are involved in mediating the clinical effect of Cop 1. Indeed, in several studies it was shown that MS patients, after receiving GA, produce mainly the Th2 type of GA-specific T cells (30, 31). It is of interest that this Th2-biased response to GA in MS patients shows cross-reactivity with MBP (32). At several levels we see cross-reactivity between GA and MBP: antibodies, T cells, and cross-triggering of cytokines. Our hypothesis is that the initiation of an autoimmune response, at least for EAE, may start with any myelin proteins, but because of their propinquity, caused most probably by inflammation, antigen spreading occurs and an anti-MBP response is provoked. This process also works in the opposite way, in this case causing bystander suppression.

MG

MS is mainly a T cell-mediated disease, whereas in MG the attack of specific antibodies on the acetylcholine receptor (AChR) is the accepted cause of disease (33). Weakness and fatigability of voluntary muscles characterize both MG and experimental autoimmune MG (EAMG) (34). Although the symptoms of MG are caused by autoantibodies produced by B cells, there is ample evidence that T cells have a key role in the etiopathology of the disease in humans and animals (34–37). Because the α-subunit of the AChR was shown to be predominant for T cell epitopes (36), we have used peptides representing different sequences of the human AChR α-subunit. Two sequences of the latter, namely peptides p195-212 and p259-271, were able to stimulate peripheral blood lymphocytes (PBL) of patients with MG (38). Furthermore, PBL of seronegative MG patients responded by either proliferation or IL-2 secretion to these peptides emphasizing the importance of AChR-specific T cells in MG (39). Peptides p195-212 and p259-271 were further shown to be immunodominant T cell epitopes in SJL and BALB/c mice, respectively (40).

Ideally the goal therapy in MG should be the elimination of autoimmune responses to the AChR specifically, without interfering with immune responses to other antigens. To this end, altered myasthenogenic peptides, which are single amino acid-substituted analogs of peptide p195-212 (207Ala) and p259-271 (262Lys), as well as a dual altered peptide ligand (APL) composed of the tandemly arranged two single peptide analogs (262Lys–207Ala), were synthesized and shown to inhibit the proliferative responses of both p195-212- and p259-271-specific T cell lines *in vitro* (41–44). The single and dual APLs were also shown to be capable of inhibiting the proliferative responses of PBL of MG patients to the myasthenogenic peptides (45). Furthermore, the analogs inhibited the *in vivo* priming to p195-212 and p259-271 (41–44).

Down-Regulation of the Proliferative Response to Torpedo AChR by the Dual APL. It was of interest to find out whether the dual APL is capable of inhibiting the proliferative responses of mice that are immunized with the multideterminant molecule of the AChR. To this end, C57BL/6 mice that are high responders to the AChR were immunized with 10 μg of the Torpedo AChR given in complete Freund's adjuvant. The mice were injected concomitantly with different doses (50–200 μg) of the dual APL given s.c. in PBS. Ten days after the immunization, lymph node cells of the mice were tested for their ability to proliferate to the immunizing Torpedo AChR. Fig. 2 shows representative results of such experiments. It can be seen that the *in vivo* treatment with the dual APL inhibited efficiently in a dose-dependent manner the ability of the lymph node cells to proliferate to the Torpedo AChR. The demonstration that the dual APL inhibited the proliferative responses of C57BL/6 mice to the Torpedo AChR indicates that the effects of the dual APL are not restricted to the strains that are high responders to the myasthenogenic peptides (e.g., BALB/c and SJL mice). Furthermore, the results suggest that the dual APL does not affect responses to the two myasthenogenic T cell epitopes only but it immunomodulates responses to other determinants within the AChR molecule, probably via epitope spreading.

Amelioration of EAMG Manifestations by the Dual APL. We have previously shown that oral administration of the dual APL to BALB/c mice afflicted with EAMG induced by the pathogenic p259-271-specific T cell line reversed EAMG manifestations in the treated mice (44). We next tested the effect of treatment with the dual APL, on an established EAMG induced in C57BL/6 susceptible mice by the Torpedo AChR. To this end, C57BL/6 mice were immunized and boosted with Torpedo AChR (20 μg per mouse). The mice diagnosed to have clinical symptoms of EAMG were divided randomly into two groups and treated three times per week either with 500 μg per mouse of the dual APL given orally in PBS or PBS only (control group). Mice were treated for 5–8 weeks. Treatment with the dual APL down-regulated significantly ($P = 0.009$) the clinical manifestations of the ongoing disease as assessed by the clinical score, grip strength (measured by a grip strength meter), and electromyography. The effects on the clinical EAMG correlated with a reduced production of anti-AChR antibody as well as a decrease in the secretion of IL-2 and, more dramatically, IFN-γ (known to play a pathogenic role in MG and EAMG) in response to AChR triggering. Thus the dual APL is an efficient immunomodulator of EAMG in mice (46).

Because rats are commonly used for the induction of EAMG, we tested the effect of treatment with the dual APL on the experimental disease induced in rats by immunization with the Torpedo AChR. To this end female Lewis rats were immunized with 50 μg of Torpedo AChR emulsified in complete Freund's adjuvant. The rats were divided into treatment groups that were either fed or injected (s.c.) with different concentrations of the

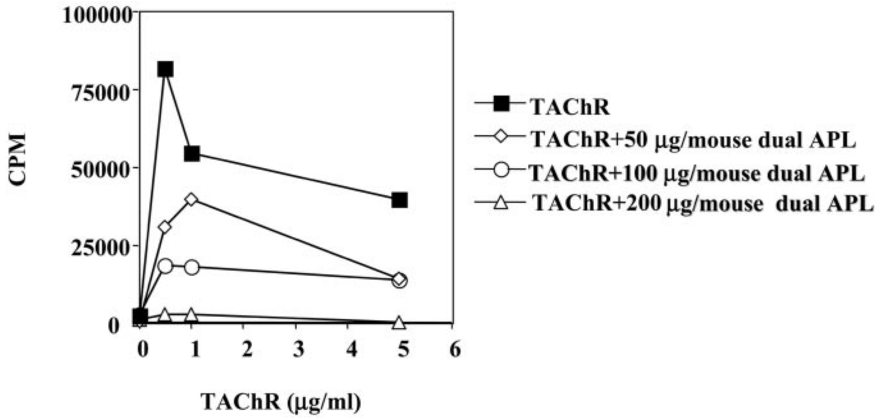

Fig. 2. The dual APL inhibits the proliferative responses of lymph node cells from C57BL/6 mice immunized with Torpedo AChR (TAChR). C57BL/6 mice were immunized with Torpedo AChR (10 μm per mouse) in complete Freund's adjuvant and were either not treated or concomitantly treated with different concentrations of the dual APL given s.c. in PBS. Ten days after immunization, popliteal lymph node cells (1×10^6) obtained from the various mouse groups were cultured in enriched medium in the presence of different concentrations of Torpedo AChR for 96 h. [^{3}H]Thymidine was then added, and 16 h later the plates were harvested and radioactivity was counted. Results are expressed as mean cpm of triplicate.

dual APL. Control groups were either fed or injected with the vehicle, namely, PBS. Treatment started at day 10 postimmunization with the Torpedo AChR and was given either twice a week (s.c. injections) or three times a week (feeding) until the end of the experiments (days 50–58 postimmunization). Treatment with the dual APL down-regulated the clinical score of the rats and up-regulated the survival of the animals. The 1-mg dose given orally and a 0.5-mg dose injected s.c. had the most significant ameliorating effects on the manifestation of the EAMG. Thus, the dual APL affects beneficially EAMG manifestations induced in rats as well.

Immunomodulation of Cytokines by the Dual APL. In an attempt to elucidate the mechanism(s) by which the dual APL down-regulates EAMG-associated responses, we first determined its effect on the cytokine profile. Thus, BALB/c or SJL mice were immunized with p259-271 and p195-212, respectively and were treated concomitantly with the dual APL administered by different routes (s.c., orally, i.p.). Ten days postimmunization splenic- and lymph node-derived lymphocytes were harvested and triggered with the immunizing myasthenogenic peptide. Culture supernatants were tested for the secreted cytokines. The results indicated that the dual APL down-regulated the secretion of the Th1-type (IL-2 and IFN-γ) cytokines by either splenocytes or lymph node cells of the treated mice. On the other hand, the secretion of IL-10 and the immunosuppressive cytokine TGF-β was up-regulated after treatment with the dual APL. The dual APL affected specifically the cytokine secretion induced by the myasthenogenic peptides because it did not have the same effect on the secretion of cytokines by immunocytes of mice that were immunized with the control antigen, namely, ovalbumin (47, 48).

Because the dual APL down-regulated the proliferation of lymph node cells of mice primed with the Torpedo AChR and because the dual APL ameliorates EAMG induced by the Torpedo AChR, its effect on the cytokine pattern in C57BL/6 mice immunized with the latter macromolecule was tested as well. Our results indicated that the s.c. administration of the dual APL caused a significant decrease in the secretion of IFN-γ, whereas secretion of TGF-β was up-regulated. As in the case of mice immunized with the myasthenogenic peptides, the administration of the dual APL to AChR injected mice down-regulated IL-2 secretion and up-regulated IL-10 secretion (47). The down-regulation of IL-2 and IFN-γ by the dual APL is of great significance because both cytokines were shown to be involved in the pathogenesis of EAMG (49–52) and MG (49, 53), and disease suppression by mucosal tolerance was associated with their down-regulation (46, 54). One of the most significant effects of the dual APL on the cytokine balance was the up-regulation of TGF-β. It was demonstrated that secretion of TGF-β was up-regulated (in parallel to down-regulation of IFN-γ secretion) in mice and rats after oral or nasal administration of AChR, and that correlated with an improvement in their clinical status (55–58). Furthermore, a correlation was found between clinical remission and increase in TGF-β expression in mononuclear cells of MG patients (59).

The Inhibitory Effects of the Dual APL on T Cell Adhesion, Matrix Metalloproteinase (MMP)-9, and Phospholipase C (PLC). To better understand the mechanism(s) underlying the *in vivo* inhibitory properties of the dual APL we analyzed T cells of mice that were immunized with a myasthenogenic peptide for their adhesiveness toward vascular cell adhesion molecule 1 (VCAM-1), as well as toward endothelial selectins. Immunization of BALB/c mice with p259-271 increased the adhesion of T cells to VCAM-1 by expanding the population of lymph node-derived T cells with high adhesiveness to the latter. Similarly, immunization with the myasthenogenic peptide enriched the T cell subsets with strong adhesiveness toward E- and P-selectins. Treatment with the dual APL (either orally or s.c.) reduced significantly the adhesiveness to VCAM-1 (60). In addition, the dual APL inhibited the myasthenogenic peptide-dependent acquisition of rolling on P- and E-selectin ligands under physiological shear flow (48). The reduced capability of T cells to interact with the endothelium after treatment with the dual APL results in reduced migratory properties of the T cells. Indeed, the role of adhesion in autoimmune diseases has been demonstrated by using anti-adhesion molecule antibody therapy in a number of autoimmune diseases (61, 62).

The expression of several MMPs was shown to be regulated by members of the integrin family (63). T cells are known to secrete MMP-2 and MMP-9 (64). The potential importance of the versatile activities of MMPs in autoimmune and inflammatory responses has been suggested by the inhibitory effects of specific MMP inhibitors, which have been shown to suppress damage to specialized tissue structures in several inflammatory and autoimmune diseases (64). In our system, MMP-2 and MMP-9 activities were measured by using gelatin zymography in lymph node-derived T cells of mice that were immunized with p259-271 and either treated or not treated with the dual APL. Immunization of BALB/c mice with p259-271 stimulated MMP-9 (but not MMP-2) activity in T cells of the immunized mice. The MMP-9 activity was markedly and specifically reduced in lymph node-derived T cells of immunized mice treated s.c. or orally with the dual APL (60). The elevated observed MMP-9 activity in p259-271-primed T cells might allow the latter autoreactive cells to degrade collagen more efficiently and pave their way through the endothelium toward the target organ. Treatment with the dual APL interferes with the latter.

The activation of PLCγ1 is a key event that leads to a spectrum of effector functions of the stimulated T cells (65). Upon T cell receptor engagement, APLs were shown to activate signal transduction events, which are distinct from those induced by the immunogenic ligand and result in different phenotypes (65, 66). Using a p259-271-specific T cell line, we demonstrated that the myasthenogenic peptide induced PLC activity upon incubation with the cells of the line, whereas the addition of the dual APL to the culture mixture selectively inhibited the induced activity (67). We therefore measured PLC activity as a marker of the T cell activation by the myasthenogenic peptides and compared PLC activity of lymph node-derived T cells from p259-271-immunized mice that were treated *in vivo* or not treated with the dual APL. The results of these experiments indicated that *in vitro* triggering of T cells from p259-271-immunized mice elevated the PLC activity significantly. However, T cells of dual APL-treated mice exhibited a reduction in PLC activity below the spontaneous levels observed in T cells that were not stimulated *in vitro* with p259-271 (60). These results show that the dual APL down-regulates migratory properties acquired by subsets of T cells after antigenic stimulation, and thus interferes with the immune response of p259-271-specific clones. The experimental efficacy of the dual APL suggests that intervention with signaling and migration-associated events might be of therapeutic potential by reducing the capability of autoreactive T cells to elicit autoreactive responses.

Treatment with the Dual APL Induces Anergy. The reproducible decrease in IL-2 secretion observed upon administration of the dual APL may suggest that at least part of its inhibitory effect is caused by its ability to cause the lymph node cells to undergo anergy. Because attempting to restore the proliferation of cells by the addition of recombinant IL-2 (rIL-2) is one of the approaches to examine this possibility, we cultured cells of SJL mice that were pretreated with the dual APL and immunized with p195-212, in the presence of different concentrations of rIL-2 and the myasthenogenic peptide. The results of repetitive experiments demonstrated that the addition of rIL-2 diminished, in a dose-dependent manner, the inhibitory effect of the dual APL. These results indicate that one of the mechanisms by which the dual APL exerts its inhibitory effects is by induction of anergy (47).

The Active Suppression of Responses Associated with EAMG by the Dual APL. Because administration of the dual APL was shown to induce anergy and, in addition to up-regulate the levels of secreted TGF-β, which was shown to be an immunosuppressive cytokine, it was of interest to determine whether the inhibitory effect can be actively transferred by cells of dual APL-treated mice. Therefore, SJL mice were treated with the dual APL (four injections every other day with 200 μg) and their splenocytes (10^7 per mouse) were injected into mice that were concomitantly immunized with p195-212. The administered cells inhibited the ability of lymph node cells of the recipient mice to proliferate to the immunizing myasthenogenic peptide. The inhibitory effect of the injected splenocytes of the dual APL-treated mice was specific, because splenocytes of PBS-treated mice did not inhibit the p195-212-specific proliferative responses (47).

Similar results were obtained when the dual APL-treated (s.c. or oral) splenocytes of C57BL/6 mice were injected to syngeneic recipients concomitant with the immunization with Torpedo AChR. Thus, splenocytes of mice administered with the dual APL inhibited significantly and specifically the proliferation to Torpedo AChR (47). The ability of splenocytes of mice that were treated with the dual APL to adoptively transfer their capacity to inhibit proliferative responses to p195-212 and Torpedo AChR, suggests that one of the mechanisms by which the dual APL exerts its inhibitory effect is by inducing an immunosuppressive response mediated by regulatory cells (68) and/or immunosuppressive cytokines like TGF-β.

The Dual APL Down-Regulates Myasthenogenic T Cell Responses by Up-Regulating CD25- and CTLA-4-Expressing T Cells. We have performed experiments to further elucidate the mechanism(s) by which the dual APL down-regulates MG-associated responses *in vivo* and characterizes the cell population(s) involved in this immunomodulatory suppressive effect. We looked into the possibility that a $CD4^+CD25^+$ cell population has a role in the down-regulating effects of the dual APL. To this end, SJL mice were either administered s.c. with the dual APL concomitant with p195-212 immunization or immunized with the myasthenogenic peptide p195-212 alone. Lymph node cells of mice of the two groups were stained for the presence of $CD4^+CD25^+$ cells and analyzed by fluorescence-activated cell sorting. When cells were taken at day 10 postimmunization, a time point at which the inhibitory effect of the dual APL can be demonstrated very clearly, an elevation in the percentage of $CD4^+CD25^+$ T cells could be demonstrated in dual APL-treated mice (12.5%), in comparison with mice immunized with p195-212 alone (10%). These results were observed in multiple experiments (69).

To determine the functional involvement of $CD4^+CD25^+$ cells in the suppressive action of the dual APL, we tested the effect of depletion of this cell population on the proliferative responses of lymph node cells of treated SJL mice. Thus, SJL mice were either injected s.c. with the dual APL concomitant with p195-212 immunization or immunized with the myasthenogenic peptide alone. Ten days after immunization, we treated the proliferative responses of lymph node cells of treated mice that were depleted of $CD25^+$ cells, in comparison with those that did not undergo any manipulation. The results demonstrated that depletion of $CD4^+CD25^+$ cell population abrogated the inhibition of proliferation exerted by the dual APL. The depletion of the $CD4^+CD25^+$ cells had an effect on the cytokine secretion by the lymph node cells. A decreased secretion of TGF-β that was associated with an elevated production of IFN-γ was observed in the population depleted of $CD4^+CD25^+$ cells. Thus, $CD4^+CD25^+$ cells play a significant role in the active suppression of the myasthenogenic-associated T cell responses by the dual APL (69).

Because the inhibitory CTLA-4 costimulatory molecule has been reported to be expressed on naturally occurring regulatory $CD4^+CD25^+$ T cells, we tested whether administration of the dual APL affects CTLA-4 expression on lymph node cells of treated SJL mice. Indeed, injections of the dual APL caused an elevation in the expression of CTLA-4 cells. To determine the functional role of the up-regulation in CTLA-4 expression, proliferative experiments were performed in the presence of anti-CTLA-4 antibodies. We could show that whereas the administration of the dual APL inhibited the proliferative responses of lymph node cells to p195-212, addition of anti-CTLA-4-neutralizing antibodies to the culture mixture abrogated the inhibitory effect and the lymph node cells proliferated efficiently to p195-212 (69).

CD28 and CTLA-4 costimulation molecules were shown to have opposite effects on T cell activation, namely, CD28 supports T cell activation and CTLA-4 mediates the termination of the immune response (70). Indeed, fluorescence-activated cell

Table 2. Summary of the effects of the dual APL

Human PBL	EAMG	Effects on EAMG-associated responses in lymph node cells		
Proliferation ↓	Autoantibodies ↓	$CD4^+CD25^+$ ↑	Proliferation ↓	MMP-9 activity ↓
IFN-γ ↓	Clinical score ↓	CTLA-4 ↑	CD28	Rolling on E- and P-selectins ↓
TGF-β ↑	Muscle strength ↑	TGF-β ↑	IL-2, IFN-γ ↓	Adherence to VCAM-1 ↓
		IL-10 ↑		PLC activity ↓

sorting analysis indicated a significant decrease in the expression of CD28 on lymph node cells of dual APL-treated mice (69). Our results regarding the above costimulatory molecules suggest that the dual APL induces the suppressive activity of the immunoregulatory T cells by up-regulating the expression of the inhibitory costimulatory molecule, CTLA-4, while decreasing the expression of the activating costimulatory molecule, CD28. Alternatively, it is possible that the up-regulation of CTLA-4 expression occurred on $CD4^+CD25^+$ cells and the down-regulation of CD28 occurred on the autoreactive T cells.

Most of the reports of $CD4^+CD25^+$ T regulatory cells are of studies performed with naïve animals (71–73). Nevertheless, results on induced immunoregulatory cells have been reported as well (74, 75). Further studies will determine whether the immunoregulatory T cells that are up-regulated by the dual APL are identical to the naturally occurring $CD4^+CD25^+$ T regulatory cells. Nevertheless, there are sufficient similarities in the phenotypes and function of these cells to raise the possibility that they derive from the same origin. Although the mechanism(s) used by the immunoregulatory cells to mediate their suppressive effect is not completely elucidated yet, the results presented here indicate that the $CD4^+CD25^+$ cells act, at least partially, through the up-regulation of the inhibitory costimulatory molecule CTLA-4. These cells may secrete TGF-β either by themselves or in a nondirect manner that triggers other cells to secrete this immunosuppressive cytokine.

Immunomodulation by the Dual APL of Autoreactive Responses of PBL of Patients with MG. We have shown previously that the dual APL inhibited the proliferative responses of PBL of MG patients to either of the myasthenogenic peptides (45). We therefore tested the ability of the dual APL to immunomodulate MG-associated responses of PBL of patients to the whole molecule of the AChR. PBL of 22 of the 27 MG patients tested responded by proliferation to Torpedo AChR. The proliferative responses of PBL of 21 of 22 responders were significantly inhibited (% mean inhibition 86.2 $\pm$ 17) by the dual APL when the latter was added to the culture mixture of cells and Torpedo AChR. The inhibition was specific because a control peptide did not inhibit these proliferative responses. The dual APL also down-regulated the levels of the secreted pathogenic cytokine IFN-γ in supernatants of stimulated PBL of 80% of the tested patients. The latter inhibitions correlated with an up-regulated production of the immunosuppressive cytokine, TGF-β. These results demonstrate that the dual APL is capable of down-regulating *in vitro* autoreactive responses of MG patients. The observed suppression is apparently via a mechanism similar to that shown by us for the animal model of EAMG. Thus, the above results suggest that the dual APL is a potential candidate for a novel specific treatment of MG patients (76).

Concluding Remarks

We have shown here that a dual APL that is based on single amino-substituted analogs of two myasthenogenic peptides is capable of down-regulating specifically *in vitro* and *in vivo* autoreactive responses that are associated with MG and EAMG. Most importantly, the dual APL was shown to ameliorate established EAMG in mice and rats. Table 2 summarizes the effects shown for the dual APL. As discussed above and shown in Table 2, the dual APL affects many functions and steps starting at earlier phases after antigenic challenge. It appears that the cumulative result of all of the cell populations and stages affected by the dual APL is the amelioration of an established EAMG.

Currently accepted treatment of MG involves agents that are both nonspecific and have multiple adverse side effects. Therefore, the use of a synthetic peptide such as the dual APL that is aimed at inhibiting specific MG-related responses without harming all other immune responses is of utmost importance. Collectively, the results of our murine *in vitro* and *in vivo* studies, and the *in vitro* inhibition by the dual APL of PBL responses of human MG patients to the AChR, suggest that the dual APL might have the desired specific therapeutic potential.

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